Different Seasonal Patterns in Song System Volume in Willow Tits and Great Tits

DIFFERENT SEASONAL PATTERNS IN SONG SYSTEM VOLUME IN WILLOW TITS AND GREAT TITS

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ABSTRACT

In most species of seasonally breeding songbirds studied to date, the brain areas that control singing (the song control system: SCS) are larger during the breeding season than at other times of the year. In the family of titmice and chickadees (Paridae), one species (the blue tit, *Cyanistes caeruleus*) shows the "traditional" pattern of seasonal changes, while another species (the black-capped chickadee, *Poecile atricapillus*) shows at best much reduced seasonal changes in the SCS. To test whether this pattern holds up in the two Parid lineages to which these two species belong, and to rule out that the differences in seasonal patterns observed were due to differences in geography or in laboratory, we compared the seasonal patterns in two song system nuclei volumes (HVC and Area X) in willow tits (*Poecile montanus*; closely related to black-capped chickadees) and great tits (*Parus major*; more closely related to blue tits) from the same area around Oulu, Finland. Both species had larger gonads in the spring than during the rest of the year. Great tit males had a larger HVC in the spring than at other times of the year, but their Area X did not change in size. Willow tits showed no seasonal change in HVC or Area X size, despite having much larger gonads in the spring than the great tits. Our findings suggest that the song system of willow tits and their relatives may be involved in learning and producing non-song social vocalizations. Since these vocalisations are used year round, there may be year-round demand on the song system. The great tit and blue tit HVC may change seasonally because demand is only placed on the song system during the breeding season, since they only produce learned vocalisations during this time. We suggest that changes were not observed in Area X because its main role is in song learning, and there is evidence that great tits do not learn new songs after their first year of life. Further study is required to determine whether our hypothesis about the role of the song system in the learned, non-song vocalisations of the willow tit and chickadee is correct, and to test our hypothesis about the role of Area X in the great tit song system.

INTRODUCTION

The song control system (SCS) of many seasonally breeding songbirds undergoes seasonal plasticity in size, as well as many other aspects of anatomy and physiology [e.g. De Groof et al., 2008, Meitzen and Thompson, 2008, Meitzen et al., 2009, Ball and Balthazart, 2010]. In the rufous-collared sparrow (*Zonotrichia capensis*), a tropical songbird, the song system is also larger when in breeding condition [Moore et al., 2004]. Typically, brain areas of the SCS, especially the nucleus HVC (used as a proper name, not an abbreviation), are larger at the time of year when birds sing the most, and these effects have been found both in the lab and in the field. However, in a few species of seasonal breeders, plasticity in the SCS has been either difficult to demonstrate or reduced in the field (wild canaries (*Serinus canaria*), [Leitner et al., 2001]; and black-capped chickadees (*Poecile atricapillus*), [Phillmore et al., 2006, Smulders et al., 2006, Phillmore et al., 2015]).

The black-capped chickadee follows the traditional seasonal songbird pattern in which males sing a courtship/territorial song in the spring breeding season, at the same time as the gonads regrow and testosterone levels increase [Smulders et al., 2006; Avey et al., 2008]. Nevertheless, in wild-caught specimens of this species, seasonal changes in the SCS have been difficult to detect [Smulders et al., 2006], except when grouping the animals by breeding condition (using testes size), rather than by season, and even then the effect was very small. In one study, the effect was restricted to the Robust nucleus of the Arcopallium (RA; [Phillmore et al., 2006]), but not HVC or Area X. In another study, a breeding condition effect was found on HVC, but not on Area X (RA was not measured in this study). In this case, the effect size was smaller than what has been observed in other songbird species [Phillmore et al., 2015], and the sample birds had been kept in captivity for a period of time. This could be problematic, since we know that captivity can have significant effects on another part of the songbird brain: the hippocampus ([Smulders et al., 2000, LaDage et al., 2009, Tarr et al., 2009, Calisi et al., 2009 (a review on the importance of differences between captive and wild species)]. Photoperiod manipulations in captivity in black-capped chickadees do result in measurable changes

50 in HVC, as well as RA and Area X [MacDougall-Shackleton et al., 2003, Phillmore et al., 2005]. The
51 seeming lack of detectable seasonal changes in the SCS of wild black-capped chickadees contrasts
52 with the easily-detectable changes of close to 60% in HVC and RA in blue tits (*Cyanistes caeruleus*), a
53 relatively closely related species [Caro et al., 2005]. Area X was not measured in this study.

54 There are a number of possible explanations for why some studies fail to find seasonal changes in
55 the SCS, while others do. It could of course be coincidence, but repeated replication of either a
56 failure to find changes, or at least of very small changes compared to other species makes that
57 explanation unlikely. It is also possible that the environment in which the studies were performed
58 matters. The results from black-capped chickadees cited above came from populations in southern
59 Ontario [Canada; Phillmore et al., 2006], Nova Scotia [Canada; Phillmore et al., 2015] and central
60 New York State [USA; Smulders et al., 2006], while the blue tit results came from Corsica [French
61 Mediterranean; Caro et al., 2005]. It is therefore possible that the species differences in seasonal
62 HVC plasticity reflect the very different environments in which these studies were conducted.

63 Alternatively, the failure to detect seasonal changes may be because there are no seasonal changes
64 (or very small ones) in those species. This could be due to an unknown event in the past that has
65 removed the plasticity to change seasonally in particular phylogenetic groups. However, this is
66 unlikely to be a good explanation, since both canaries (at least domesticated ones, [Nottebohm,
67 1981]) and black-capped chickadees still show seasonal changes when tested in captivity
68 [Macdougall-Shackleton et al., 2003]. This suggests that the potential for seasonal changes is present
69 in these species. So why do we not see seasonal changes in the field? Even though wild canaries
70 change their repertoire across seasons, they do not change their singing intensity, singing (and
71 therefore using their SCS) year-round [Leitner et al., 2001]. If year-round use of the SCS is associated
72 with a lack of seasonal changes in SCS volumes, then one potential explanation for the Parid
73 situation is that the species differences could reflect the different vocalization repertoires and
74 seasonal uses of these repertoires in the two species: chickadees have a complex set of learned calls,

75 which are used year-round by both sexes, and a relatively simple breeding song [Avey et al., 2008];
76 while blue tits have a more complex song and lack the complex social calls of the chickadees [Bijnens
77 and Dhondt, 1984]. This would suggest chickadees and their relatives use the SCS year-round, while
78 blue tits and their relatives don't.

79 The current study aims to eliminate the possibility that different environments cause differences in
80 seasonal patterns, and to verify that the different patterns observed in chickadees and blue tits are
81 not specific to those species, but generalize to other species in their clades. We compared the
82 seasonal plasticity of two SCS nuclei, HVC and Area X, in two species exhibiting a different seasonal
83 pattern of vocalizations, but which were collected from the same environment: willow tits (*Poecile*
84 *montanus*) and great tits (*Parus major*).

85 Willow tits and great tits are sedentary hole-nesting passerines. In the Oulu area of northern Finland
86 (ca. 65°3'N, 25°27'E, average elevation 15 meters), the main singing period for great tits and willow
87 tits occurs in March and April when the breeding territories are established. The laying time of
88 genuine first clutches is May; the annual median onset of egg-laying range from 5th to 20th May in
89 the willow tit and from 7th to 30th May in the great tit [Vatka et al. 2011, 2014]. The earliest willow tit
90 and great tit nests have been commenced on 29 and 30 April, respectively. In both species the
91 earliest clutches start hatching in late May and the main nestling period is in June and early July.
92 Only females incubate, but both parents provide parental care for young. Both species can lay repeat
93 clutches if the first nesting fails. Parents go through postnuptial molt, willow tits in June–August and
94 great tits in late June–October [Orell & Ojanen, 1980].

95 Willow tits are closely related to the black-capped chickadee. They winter at their breeding grounds
96 in small, highly territorial and stable groups [Koivula & Orell, 1988, Ekman, 1989]. They have a set of
97 complex social vocalizations, which they use year-round, similarly to the chickadee [Haftorn, 1993,
98 Ficken et al., 1978, Ficken et al., 1985, Ficken et al., 1987, Miyasato and Baker, 1999, Baker et al.,
99 2000]. These non-song vocalisations have been shown to be learned, at least in the chickadee

[Mammen and Nowicki, 1981, Ficken and Weise, 1984, Ficken et al., 1987, Nowicki, 1989, Shackleton et al., 1992, Kroodsma et al., 1995, Hughes et al., 1998]. Their territorial song, on the other hand, is relatively simple compared to most songbird songs [Martens and Nazarenko, 1993].

Great tits are more closely related to blue tits, and equally lack the social vocalizations of the chickadee [Johansson et al., 2013]. They follow the "traditional" songbird pattern of singing a complex courtship and territorial song during the breeding season [Rost, 1990]. Great tits overwinter in constantly changing non-territorial flocks outside their breeding territories [Ekman, 1989]. If the production of song or song-like vocalisations is a potential mechanism of seasonal changes in the SCS, then we should find seasonal changes in the SCS of great tits, but not of willow tits, even when both are collected from the same environment.

MATERIALS AND METHODS

Animals

Subjects were adult male great tits and willow tits, captured at Oulu under a licence from the North Osthrobothnian Regional Environmental Centre. The birds were captured in two seasons: 2006-2007 and during April 2015. Great tits were caught using funnel traps baited with food, and willow tits were caught using mist nets, song playback and decoy birds. All birds were aged in the hand based on plumage. Great tits were sexed using the colour and pattern of their plumage, and wing length if necessary, and willow tits by the observation of song production and wing length. Sex was confirmed after the dissection of the gonads.

		Number of samples collected						
		Breeding season		**Rest of the year**				
		March	April	Aug	Sept	Nov	Dec	***Total***
Species	Great tit	3	6	4	3	2	/	18
	Willow tit	/	10	3	4	1	1	19
	Total	19		18				**37**

Table 1: total sample sizes from 2006/7 and 2015 of great tit and willow tit brains collected in the breeding season (March or April) or the rest of the year (August, September, November or December).

2006 – 2007

In the spring breeding season, male great tits were collected between 24th March and 30th March 2007, and willow tits were collected between 16th April and 22nd April 2007. The average timing of the first clutches in 2007 was the 15th May for great tits, and the 10th May for willow tits. During this sampling period, our sample from the breeding season consisted of 8 birds: 3 great tits and 5 willow tits, and our sample from the rest of the year consisted of 18 birds: 9 great tits and 9 willow tits. For Area X analyses, 1 great tit sample and 3 willow tit samples were not included because of tissue damage.

April 2015

To increase our breeding season sample size, more birds were collected in April 2015. Great tits were collected between 8th April and 16th April, and willow tits were collected between 16th April and 22nd April. The average timing of the first clutches in 2015 was the 14th May for great tits, and the 10th May for willow tits. Our sample from this period consisted of 11 birds: 6 great tits and 5 willow tits. Our exact sample sizes are indicated in Table 1.

Validating breeding condition

To assess whether the birds were in breeding condition at the time of capture, their gonads were weighed after the birds had been humanely killed and the brain dissection had been performed. In

2006/07 gonads were rapidly frozen on dry ice after dissection. They were then shipped and weighed back in Newcastle. To quantify gonad size, the frozen gonads were weighed in their centrifuge tubes. The weight of the same empty frozen centrifuge tube was then subtracted from all of these weights. In 2015, gonads were weighed fresh after dissection in Finland, and then discarded.

Histology

2006 – 2007

Birds were killed with rapid decapitation. One hemisphere of the brain was immersed in 4% formaldehyde in PBS. After 48 hours of fixation, the hemispheres were cryoprotected in 30% sucrose solution, embedded in O.C.T. (Optimal Cutting Temperature compound for cryosectioning), frozen on dry ice and stored at -80°C. After all of the samples had been collected, they were shipped from Oulu to Newcastle. They were all sectioned at 70 µm on a cryostat, and every other section was thaw-mounted onto gelatin-coated slides. The sections were stained with cresyl violet and coverslipped.

April 2015

Birds were anaesthetised using isoflurane before decapitation. Both hemispheres of the brain were immersed in a solution of 4% formaldehyde in PBS. After 48 hours of fixation, the brains were cryoprotected in 30% sucrose solution and stored in a cold room at 4°C. After all of the samples had been collected, they were shipped from Oulu to Newcastle for further processing and histological measurements. They were embedded in O.C.T., sectioned at 40 µm on a cryostat into PBS solution. Every other free floating section was mounted onto gelatin-coated slides. The sections were then stained with cresyl violet and coverslipped.

Brain region morphometry

To quantify the size of the brain areas we were interested in, we outlined the nuclei in all of the sections in which they could be seen. For HVC, nucleus Rotundus (Rt) and Telencephalon measurements, we used StereoInvestigator® connected to a Leica DMLB microscope with a Prior automated stage and an Optronics Microfire digital camera. For Area X measurements, we used Zen® connected to a Nikon Eclipse microscope with a rotatable stage and a Zeiss Axiocam 105 colour camera. Figure 1 displays representative examples of the nuclei we outlined in our morphometric analyses. Outlines were drawn using a 2.5x or 2x objective, sometimes changing to the 10x objective for clarification of boundaries. Only half brains were collected in 2006/07 (equal numbers of left and right hemispheres), but we collected whole brains in 2015. For consistency, we outlined only one hemisphere in the brains collected in 2015. We outlined equal numbers of left and right hemispheres, which were randomly allocated beforehand.

Rt and the telencephalon (both used as control areas in our analysis) were outlined by different people for the 2006-2007 samples vs. the 2015 samples. All HVC and Area X outlines were performed by the same person (GKL). The outlines of the 2006/07 samples were performed blind to species and season. It was not possible to be blind to the season of the 2015 samples since they were all collected at the same time of year, however the outlines were performed blind to species.

2006 – 2007 (70 µm sections, every other section taken)

To calculate the volume of HVC, Area X and Rt, the area of each section was multiplied by 140 µm (the distance between measurements). These volumes were added up for all the sections containing the nucleus of interest. To calculate telencephalon volume, its surface area was measured on every 4th section on the slides, multiplied by 560 µm and added up.

April 2015 (40 µm sections, every other section taken)

To calculate the volume of HVC, Area X and Rt, the area of each section was multiplied by 80 µm. These volumes were added up for all the sections containing the nucleus of interest. To calculate

telencephalon volume, its surface area was measured on every 14th section on the slides. This was then multiplied by 560 µm. To provide a starting point and standardization of measurement, the first section where the anterior commissure was present was always one of the sections measured.

Data analysis

We investigated differences in seasonal patterns between species by testing whether an interaction between season and species could explain variation in volumes of HVC, Area X and Rt, and in gonad weight. All measurements were natural-log-transformed for data analysis (+ 1 to avoid negative scaling). The design of our statistical models are classic factorial AN(C)OVAs. We implemented these in the Generalized Linear Model function in SPSS version 22 for Windows, with a linear outcome variable because this gave us a more flexible output, enabling pairwise comparisons between means. The output from these models is Wald's χ^2. All analyses were also run as classic AN(C)OVAs and the outcomes were qualitatively the same.

Tests for HVC, Area X and Rt were run using two factors: species (willow tit/great tit) and season (breeding season/rest of the year), and we tested for main effects of species and season, as well as for the interaction between these factors. We included the volume of the telencephalon in the model as a covariate, to control for any overall size differences between the samples because of the two different methods used to process the tissue (see [Smulders 2002]). We included telencephalon as a co-variate rather than analysing each nucleus as a percentage of the telencephalon, because ratios conflate variation in the numerator with variation in the denominator. However, we will plot our results as percentages of telencephalon, to enable readers to compare our results with other studies which have used these ratios in their analyses of nuclei volume.

No other factors or interactions between factors and co-variate were included in the model. Results were considered significant if $p < .05$.

RESULTS

Gonad mass

We measured gonad mass as a proxy of breeding condition. In both species, testes were larger in birds caught during the spring (March and April) than during the rest of the year (August – December; χ^2_1 = 96.3, p <0.001). We also found a significant interaction between species and season (χ^2_1 = 6.17, p = 0.013; see Figure 2). During the spring, willow tits had larger testes than great tits (p = 0.003). This effect was not present during the rest of the year, when birds were not in breeding condition (p = 0.593).

Brain morphometry

We checked our calculation of nuclei volume was consistent with another measure used in the literature: the formula for a cone frustum (see [Smith et al., 1995]). We observed similar results and levels of significance, and the two measures were significantly correlated (r = 0.944, p <0.001). The analyses we report below use our original calculation of volume.

HVC volume

We investigated whether the volume of the SCS nucleus HVC changed seasonally in both great tits and willow tits. Telencephalon volume significantly predicted HVC volume (χ^2_1 = 42.84, p < 0.001) and there were no independent main effects of species (χ^2_1 = 2.32, p = 0.128) or season (χ^2_1 = 2.434, p = 0.119). There was, however, a significant interaction between species and season (χ^2_1 = 6.56, p = 0.01; Figure 3). In great tits, HVC volume was larger in the spring, than when birds were not in breeding condition (p = 0.006). There was no seasonal difference in HVC volume in the willow tits (p = 0.652). Comparing the species within each breeding season, we find that breeding great tits have significantly larger HVC volumes than breeding willow tits (p = 0.004), but this effect was not present outside of the spring (p = 0.498).

Area X volume

We also investigated whether the volume of the SCS nucleus Area X changed seasonally in both great tits and willow tits. Telencephalon volume significantly predicted Area X volume ($\chi^2_1 = 64.92$, $p < 0.001$) and there were no independent main effects of species ($\chi^2_1 = 0.095$, $p = 0.758$) or season ($\chi^2_1 = 0.005$, $p = 0.944$). In contrast to our analysis of HVC, we found no significant interaction between species and season ($\chi^2_1 = 0.570$, $p = 0.450$; Figure 3).

Nucleus rotundus volume

To ensure that the seasonal effect of the increase in HVC volume was specifically in the SCS, we measured a control structure which is not involved in song control: the visual nucleus Rotundus (Rt; [Laverghetta & Shimizu, 1999]). It is also easy to identify, making the quantification of its volume reliable and repeatable across individuals.

Telencephalon volume significantly predicted Rt volume ($\chi^2_1 = 182.73$, $p < 0.001$).As expected, we found no evidence of an effect of season on Rt volume ($\chi^2_1 = 1.76$, $p = .185$), nor any interaction between species and season ($\chi^2_1 = 0.17$, $p = 0.679$). However, there was a significant main effect of species: Rt was larger relative to telencephalon in great tits than in willow tits ($\chi^2_1 = 6.08$, $p = 0.014$; Figure 3).

DISCUSSION

Main findings

Our results suggest seasonal stability in the size of the willow tit SCS, compared to seasonal plasticity in the size of HVC in the great tit. The lack of seasonal change in the willow tit SCS in the field is consistent with previous studies on the black-capped chickadee, a closely-related species, which have reported either reduced seasonal change or seasonal stability in the SCS [Phillmore et al., 2006, Smulders et al., 2006, Phillmore et al., 2015]. Ecology and behaviour of willow tits and black-capped chickadees are very similar to each other. The finding of changes in HVC size in early spring in great tits is also consistent with previous findings from blue tits [Caro et al., 2005]. Our results give support

to the hypothesis that the difference in seasonal plasticity between these two species is related to the differences in vocalizations made by the two groups of birds [Smulders et al., 2006]. These differences in turn relate to differences in winter social systems.

Functional explanations of seasonal stability

Although we measured the size of the SCS nuclei, previous research has observed seasonal changes using alternative measures, including neuron number, neuronal density and incorporation of new neurons into the nucleus (see [Tramontin & Brenowitz, 2000] for a review). One explanation for our findings is that measuring volume was not sensitive enough to observe subtle seasonal changes in our sample of birds. A previous study of the black-capped chickadee did find small seasonal changes in the SCS, which were of a smaller magnitude to the changes observed in the majority of species [Phillmore et al., 2015]. Measuring neuron number or neuronal density in our samples could therefore yield different results than our measure of volume.

Nevertheless, in most songbirds, SCS volumes do change seasonally. We first consider why HVC volume changes seasonally in great tits, but not in willow tits. Willow tits are closely related to black-capped chickadees, and have a similar large and complex repertoire of social calls, which they perform year-round [Haftorn, 1993]. Great tits, in contrast, are more closely related to blue tits, and equally lack the learned social vocalizations, while possessing a complex courtship/territorial song, like most other seasonally breeding songbirds studied [McGregor and Krebs, 1982]. Given these differences in call repertoire and in the seasonal pattern of vocalizations, we hypothesise that HVC is responsible for the learning and generation of the complex social call vocalizations year-round, just like it plays a role in the learned zebra finch long call [Simpson and Vicario, 1990]. The extra singing during the breeding season then does not add sufficient demand on HVC's circuitry to lead to a large increase in volume during the breeding season. Interestingly, the size of the willow tit HVC is equivalent to the great tit non-breeding HVC, and smaller than the breeding HVC. If our hypothesis is

286 correct, this suggests that the demands on HVC's circuitry are less for the learned social vocalizations
287 and simple song than they are for the complex great tit song.

288 In contrast to our results for HVC, we found no evidence of seasonal plasticity in Area X in either
289 species. The lack of seasonal plasticity in Area X volume in the willow tits is consistent with the lack
290 of seasonal plasticity in HVC. The finding is also consistent with several other studies on seasonal
291 songbirds which have found evidence of plasticity in one or two SCS nuclei (usually HVC) but not others.
292 Although the study on blue tits did not measure Area X [Caro et al., 2005], two studies of the black-
293 capped chickadee have found small seasonal plasticity in HVC but not in their Area X [Phillmore et al.,
294 2006, Phillmore et al., 2015]. HVC is involved in the motor production of song as well as in song
295 learning, whereas Area X is involved in learning, but not production. If great tits learn their song types
296 during an early life critical period and lose the ability to do so afterwards (close-ended song learning,
297 see [Marler., 1970]), this could explain why we have observed seasonal changes in the great tit HVC,
298 but not in Area X. However, there is not a definite consensus about when great tits learn their song.
299 Previous observations have suggested that great tits may learn songs from neighbours in adulthood
300 [McGregor & Avery., 1985, McGregor & Krebs., 1989, Franco & Slabbekoorn., 2009], while a recent
301 study suggests that these findings were due to methodological issues, and that great tit is actually a
302 close-ended learner [Rivera-Gutierrez et al., 2011].

303 To our knowledge, our study is the first to investigate seasonal changes in the great tit SCS. Additional
304 studies are required to understand the lack of changes we have observed in Area X, and to determine
305 when great tits learn their song. The fact that seasonal changes have been reported in HVC and RA,
306 but not in Area X in the white-crowned sparrow (*Zonotrichia leucophrys)*, a confirmed close-ended
307 learner, supports our hypothesis [Smith et al., 1995, Smith et al., 1997, Tramontin et al., 1998]. Growth
308 of Area X has only been reported in this species after photoperiodic manipulations in experimental
309 settings [Brenowitz et al., 1998, Thompson & Brenowitz 2005]. Although we were unable to measure
310 SCS nucleus RA because of issues with our older samples' tissue quality, determining whether it

changes seasonally in the great tit could also add strength to our hypothesis about Area X, since RA is mainly involved in motor production, which does change seasonally.

The mechanisms of seasonal stability and plasticity

The pattern we observed in our data is consistent with the idea that HVC size is driven by the amount of vocalization performed by the birds [Sartor et al., 2005]. In great tits, the complex song is only used intensively during the breeding season. This change in usage of the motor circuit could then drive the change in HVC size. The social vocalizations in willow tits and chickadees, however, are used intensively year-round [Avey et al., 2008], resulting in seasonal stability in HVC size. The fact that breeding-condition-related changes in the SCS were detected in captive black-capped chickadees [MacDougall-Shackleton et al., 2003] supports this argument. Captive chickadees produce the same vocalizations as wild birds, but in much smaller quantities [Avey et al., 2011]. If vocal activity is indeed responsible for the size of the SCS, then the lower level of vocalizations in captivity might lead to a smaller SCS when birds are not in breeding condition (including Area X in that case; [MacDougall-Shackleton et al., 2003]). The increase in fee-bee singing observed under increasing photoperiod in the lab may then be a large enough change in the use of learned vocalizations to have a detectable effect on SCS volumes, because the baseline vocal activity is so much lower than in the field. This mechanism may also apply to other groups of songbirds, such as the European starling, where direct effects of singing activity on SCS have been reported [Ball et al., 2004, Sartor & Ball., 2005].

The fact that HVC did not change in size in the willow tit, despite their large testes and their presumably high levels of testosterone in the spring, suggests that in this species there is not a direct effect of testosterone on HVC volume, as there is in other species [Brenowitz and Lent, 2000]. Although we did not directly measure testosterone in our population, several other studies have found seasonal changes in testosterone levels in the willow tit [Silverin., 1984, Silverin et al., 1986], and the great tit (plasma testosterone: [Van Duyse et al., 2003], testosterone metabolizing enzymes in the brain: [Silverin & Deviche, 1991]), which suggests that there is a change in testosterone in our study

species during the breeding season which matches the change in gonad size that we have observed. Interestingly, in blue tits, evidence also suggests that testosterone is not an important factor in driving SCS seasonal plasticity, as SCS nuclei increase in size before the spring surge in testosterone [Caro et al. 2005]. This may mean that in Parids in general, direct effects of testosterone on seasonal changes in SCS nuclei size are less likely. This is similar to red-backed fairy wrens (*Malurus melanocephalus*) in which a dissociation between testosterone levels and SCS nuclei size has been reported [Schwabl et al., 2015], but unlike other groups of songbirds, where testosterone seems to be the driving force behind seasonal plasticity in the SCS (e.g. the rufous collared sparrow [Small et al., 2015] and the canary [Madison et al., 2015], see [Tramontin & Brenowitz, 2000] for a review). Additional studies of seasonal changes in the Parid song system which directly measure and manipulate testosterone levels (both systemically and locally [Tramontin et al., 2000, Brenowitz et al., 2007, Meitzen et al., 2007]) are necessary to determine whether our hypothesis is correct.

Conclusion

In conclusion, this study is the first to directly compare seasonal changes in the song control system between two Parid species from the same environment, exhibiting differences in song behaviour. Our results confirm the lack of seasonal changes in HVC and Area X in the "atypical" species which produces complex social vocalisations year-round in addition to its simple courtship song, and the existence of seasonal plasticity in the HVC of the more "traditional" species, which has a much smaller, simpler repertoire of non-song vocalisations, but a more complex courtship song. We suggest that the willow tit HVC and Area X are stable in size throughout the year in the field because these nuclei are involved in the learning and production of its social vocalizations, as well as its courtship song. Area X may not change seasonally in the great tit because they are potentially close-ended learners. Direct study of the role of HVC and Area X in the song and non-song vocalizations in different Parid species will be required to test our hypotheses.

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366 **REFERENCES**

367 Avey, M T, Quince, A F & Sturdy, C B (2008). Seasonal and diurnal patterns of black-capped chickadee
368 (*Poecile atricapillus*) vocal production. Behav Process*,* 77: 149-155.

369 Avey, M T, Rodriguez, A & Sturdy, C B (2011). Seasonal variation of vocal behaviour in a temperate
370 songbird: Assessing the effects of laboratory housing on wild-caught, seasonally breeding
371 birds. Behav Process*,* 88: 177-183.

372 Baker, M C, Howard, T M & Sweet, P W (2000). Microgeographic variation and sharing of the gargle
373 vocalization and its component syllables in black-capped chickadee (Aves, Paridae, *Poecile*
374 *atricapillus*) populations. Ethology*,* 106: 819-838.

375 Ball, G F, Auger, C J, Bernard, D J, Charlier, T D, Sartor, J J, Riters, L V, & Balthazart, J (2004). Seasonal
376 plasticity in the song control system: multiple brain sites of steroid hormone action and the
377 importance of variation in song behavior. Annals of the New York Academy of Sciences,
378 1016(1): 586-610.

379 Ball, G F & Balthazart, J (2010). Seasonal and hormonal modulation of neurotransmitter systems in the
380 song control circuit. Journal of Chemical Neuroanatomy*,* 39: 82-95.

381 Bernard, D J, & Ball, G F (1997). Photoperiodic condition modulates the effects of testosterone on song
382 control nuclei volumes in male European starlings. General and comparative endocrinology,
383 105(2): 276-283.

384 Bijnens, L & Dhondt, A A (1984). Vocalizations in a Belgian blue tit, *Parus c. caeruleus*, population. De
385 Giervalk*,* 74: 243-269.

386 Brenowitz, E A, & Lent, K (2002). Act locally and think globally: intracerebral testosterone implants
387 induce seasonal-like growth of adult avian song control circuits. *Proceedings of the National*
388 *Academy of Sciences*,*99*(19), 12421-12426.

389 Brenowitz, E A, Lent, K, & Rubel, E W (2007). Auditory feedback and song production do not regulate
390 seasonal growth of song control circuits in adult white-crowned sparrows. The Journal of
391 neuroscience, 27(25), 6810-6814.

392 Calisi, R M, & Bentley, G E (2009). Lab and field experiments: are they the same animal?. Hormones
393 and Behavior, 56(1), 1-10.
394 Caro, S P, Lambrechts, M A & Balthazart, J B (2005). Early seasonal development of brain song control
395 nuclei in male blue tits. Neurosci Lett, 386: 139-144.
396 De Groof, G, Verhoye, M, Van Meir, V, Balthazart, J & Van Der Linden, A (2008). Seasonal rewiring of
397 the songbird brain: An in vivo MRI study. Eur J Neurosci, 28: 2475-2485.
398 Ekman, J (1989). Ecology of non-breeding social systems of *Parus*. Wilson Bull, 101: 263-288.
399 Ficken, M S, Ficken, R W & Apel, K M (1985). Dialects in a call associated with pair interactions in the
400 black-capped chickadee. Auk, 102: 145-151.
401 Ficken, M S, Ficken, R W & Witkin, S R (1978). Vocal repertoire of the black-capped chickadee. Auk, 95:
402 34-48.
403 Ficken, M S & Weise, C M (1984). A complex call of the black-capped chickadee (*Parus atricapillus*). I.
404 Microgeographic variation. Auk, 101: 349-360.
405 Ficken, M S, Weise, C M & Reinartz, J A (1987). A complex vocalization of the black-capped chickadee.
406 II. Repertoires, dominance and dialects. Condor, 89: 500-509.
407 Haftorn, S (1993). Ontogeny of the Vocal Repertoire in the Willow Tit Parus montanus. Ornis Scand,
408 24: 267-289.
409 Hughes, M, Nowicki, S & Lohr, B (1998). Call learning in black-capped chickadees (*Parus atricapillus*):
410 The role of experience in the development of 'chick-a-dee' calls. Ethology, 104: 232-249.
411 Johansson, U S, Ekman, J, Bowie, R C, Halvarsson, P, Ohlson, J I, Price, T D, & Ericson, P G (2013). A
412 complete multilocus species phylogeny of the tits and chickadees (Aves: Paridae). Molecular
413 phylogenetics and evolution, 69(3), 852-860.
414 Koivula, K, Orell, M (1988) Social rank and winter survival in the Willow Tit Parus montanus. Ornis
415 Fennica, 65, 114–120.
416 Kroodsma, D E, Albano, D J, Houlihan, P W & Wells, J A (1995). Song development by black-capped
417 chickadees (*Parus atricapillus*) and carolina chickadees (*P. carolinensis*). Auk, 112: 29-43.

LaDage, L D, Roth II, T C, Fox, R A, & Pravosudov, V V (2009). Effects of captivity and memory-based experiences on the hippocampus in mountain chickadees. Behavioral neuroscience, 123(2): 284.

Laverghetta, A V, & Shimizu, T (1999). Visual discrimination in the pigeon (*Columba livia*): effects of selective lesions of the nucleus rotundus. Neuroreport, 10(5), 981-985.

Leitner, S, Voigt, C, Garcia-Segura, L M, Van't Hof, T & Gahr, M (2001). Seasonal activation and inactivation of song motor memories in wild canaries is not reflected in neuroanatomical changes of forebrain song areas. Hormones & Behavior, 40: 160-168.

Leitner, S, Voigt, C, & Gahr, M (2001). Seasonal changes in the song pattern of the non-domesticated island canary (Serinus canaria) a field study. Behaviour, 138(7), 885-904.

MacDougall-Shackleton, S A, Hernandez, A M, Valyear, K F & Clark, A P (2003). Photostimulation induces rapid growth of song-control brain regions in male and female chickadees (*Poecile atricapilla*). Neurosci Lett, 340: 165-168.

Madison, F N, Rouse, M L, Balthazart, J, & Ball, G F (2015). Reversing song behavior phenotype: Testosterone driven induction of singing and measures of song quality in adult male and female canaries (*Serinus canaria*). General and comparative endocrinology, 215: 61-75.

Mammen, D L & Nowicki, S (1981). Individual differences and within-flock convergence in chickadee calls. Behav Ecol Sociobiol, 9: 179-186.

Marler, P (1970). A comparative approach to vocal learning: song development in White-crowned Sparrows. Journal of comparative and physiological psychology, 71(2p2), 1.

Martens, J & Nazarenko, A A (1993). Microevolution of eastern palaearctic grey tits as indicated by their vocalizations (Parus [Poecile] Paridae, Aves). I. *Parus montanus*: Contributions to the Fauna of the Far East, No. 2. Journal of Zoological Systematics and Evolutionary Research, 31: 127-143.

McGregor, P K, & Avery, M I (1986). The unsung songs of great tits (Parus major): learning neighbours' songs for discrimination. Behavioral Ecology and Sociobiology, 18(4), 311-316.

McGregor, P K & Krebs, J R (1982). Song types in a population of great tits (*Parus major*): their distribution, abundance and acquisition by individuals. Behaviour, 79: 126-152.

Meitzen, J & Thompson, C K (2008). Seasonal-like growth and regression of the avian song control system: Neural and behavioral plasticity in adult male Gambel's white-crowned sparrows. Gen Comp Endocrinol, 157: 259-265.

Meitzen, J, Moore, I T, Lent, K, Brenowitz, E A, & Perkel, D J (2007). Steroid hormones act transsynaptically within the forebrain to regulate neuronal phenotype and song stereotypy. The Journal of Neuroscience, 27(44), 12045-12057.

Meitzen, J, Weaver, A L, Brenowitz, E A & Perkel, D J (2009). Plastic and stable electrophysiological properties of adult avian forebrain song-control neurons Across changing breeding conditions. J Neurosci, 29: 6558-6567.

Miyasato, L E & Baker, M C (1999). Black-capped chickadee call dialects along a continuous habitat corridor. Anim Behav, 57: 1311-1318.

Moore, I T, Wingfield, J C, & Brenowitz, E A (2004). Plasticity of the avian song control system in response to localized environmental cues in an equatorial songbird. The Journal of neuroscience, 24(45): 10182-10185.

Nottebohm, F (1981). A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. Science, 214(4527), 1368-1370.

Nottebohm, F, Nottebohm, M E, & Crane, L (1986). Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. Behavioral and neural biology, 46(3), 445-471.

Nowicki, S (1989). Vocal plasticity in captive black-capped chickadees: the acoustic basis and rate of call convergence. Anim Behav, 37: 64-73.

Orell, M & Ojanen, M (1980) Overlap between breeding and moulting in the great tit Parus major and the willow tit P. montanus in northern Finland. – Ornis Scand. 11:43-49.

469 Orell, M & Ojanen, M (1983). Timing and length of the breeding season of the great tit (*Parus major)*
470 and the willow tit (*P. montanus)* near Oulu, northern Finland. Ardea 71:183-198.
471 Phillmore, L S, Hoshooley, J S, Hahn, T P, & MacDougall-Shackleton, S A (2005). A test of absolute
472 photorefractoriness and photo-induced neural plasticity of song-control regions in black-
473 capped chickadees (*Poecile atricapillus*). Canadian journal of zoology, 83(5): 747-753.
474 Phillmore, L S, Hoshooley, J S, Sherry, D F & MacDougall-Shackleton, S A (2006). Annual cycle of the
475 black-capped chickadee: seasonality of singing rates and vocal-control brain regions. J
476 Neurobiol*,* 66: 1002-10.
477 Phillmore, L S, MacGillivray, H L, Wilson, K R, & Martin, S (2015). Effects of sex and seasonality on the
478 song control system and FoxP2 protein expression in black-capped chickadees (*Poecile*
479 *atricapillus*). Developmental neurobiology, 75(2): 203-216.
480 Rivera-Gutierrez, H F, Pinxten, R, & Eens, M (2011). Difficulties when assessing birdsong learning
481 programmes under field conditions: a re-evaluation of song repertoire flexibility in the great
482 tit. PloS one, 6(1), e16003.
483 Rost, R (1990). Hormones and behaviour. A joint examination of studies on seasonal variation in song
484 production and plasma levels of testosterone in the great tit *Parus major*. J Ornithol*,* 131: 403-
485 412.
486 Sartor, J J, & Ball, G F (2005). Social suppression of song is associated with a reduction in volume of a
487 song-control nucleus in European starlings (*Sturnus vulgaris*). Behavioral neuroscience, 119(1):
488 233.
489 Sartor, J J, Balthazart, J & Ball, G F (2005). Coordinated and dissociated effects of testosterone on
490 singing behavior and song control nuclei in canaries (*Serinus canaria*). Horm Behav*,* 47: 467-
491 76.
492 Schwabl, H, Dowling, J, Baldassarre, D T, Gahr, M, Lindsay, W R, & Webster, M S (2015). Variation in
493 song system anatomy and androgen levels does not correspond to song characteristics in a
494 tropical songbird. Animal Behaviour, 104: 39-50.

495 Shackleton, S A, Ratcliffe, L & Weary, D M (1992). Relative frequency parameters and song recognition
496 in black-capped chickadees. Condor, 94: 782-785.
497 Silverin, B (1984). Annual gonadotropin and testosterone cycles in free-living male birds. Journal of
498 Experimental Zoology, 232(3), 581-587.
499 Silverin, B, Viebke, P A, & Westin, J (1986). Seasonal changes in plasma levels of LH and gonadal
500 steroids in free-living willow tits Parus montanus. Ornis Scandinavica, 230-236.
501 Silverin, B, & Deviche, P (1991). Biochemical characterization and seasonal changes in the
502 concentration of testosterone-metabolizing enzymes in the European great tit (Parus major)
503 brain. General and comparative endocrinology, 81(1), 146-159.
504 Simpson, H B & Vicario, D S (1990). Brain pathways for learned and unlearned vocalizations differ in
505 zebra finches. J Neurosci, 10: 1541-56.
506 Small, T W, Brenowitz, E A, Wojtenek, W, & Moore, I T (2015). Testosterone Mediates Seasonal Growth
507 of the Song Control Nuclei in a Tropical Bird. Brain, behavior and evolution, 86(2): 110-121.
508 Svensson, L (1992) Identification Guide to European Passerines. British Trust for Ornithology.
509 Tramontin, A D & E A Brenowitz (2000). Seasonal plasticity in the adult brain. Trends Neurosci. 23:
510 251–258.
511 Tramontin, A D, Hartman, V N, & Brenowitz, E A (2000). Breeding conditions induce rapid and
512 sequential growth in adult avian song control circuits: a model of seasonal plasticity in the
513 brain. The Journal of Neuroscience, 20(2), 854-861.
514 Smith, G T, Brenowitz, E A, Wingfield, J C, & Baptista, L F (1995). Seasonal changes in song nuclei and
515 song behavior in Gambel's white-crowned sparrows. Journal of neurobiology, 28(1), 114-125.
516 Smulders, T V, Casto, J M, Nolan, V, Ketterson, E D, & DeVoogd, T J (2000). Effects of captivity and
517 testosterone on the volumes of four brain regions in the dark-eyed junco (*Junco hyemalis*).
518 Journal of neurobiology, 43(3): 244-253.

519 Smulders, T V (2002). Natural breeding conditions and artificial increases in testosterone have
520 opposite effects on the brains of adult male songbirds: a meta-analysis. Hormones and
521 behavior, 41(2): 156-169.
522 Smulders, T V, Lisi, M D, Tricomi, E, Otter, K A, Chruszcz, B, Ratcliffe, L M & DeVoogd, T J (2006). Failure
523 to detect seasonal changes in the song system nuclei of the black-capped chickadee (*Poecile*
524 *atricapillus*). J Neurobiol*,* 66: 991-1001.
525 Tarr, B A, Rabinowitz, J S, Imtiaz, M A, & DeVoogd, T J (2009). Captivity reduces hippocampal volume
526 but not survival of new cells in a food-storing bird. Developmental neurobiology, 69(14): 972-
527 981.
528 Van Duyse, E, Pinxten, R, & Eens, M (2003). Seasonal fluctuations in plasma testosterone levels and
529 diurnal song activity in free-living male great tits. General and comparative endocrinology,
530 134(1), 1-9.
531 Vatka, E, Orell, M, Rytkönen, S (2011) Warming climate advances breeding and improves synchrony
532 of food demand and food availability in a boreal passerine. Global Change Biology, 17, 3002–
533 3009.
534 Vatka, E, Rytkönen, S, Orell, M (2014) Does the temporal mismatch hypothesis match in boreal
535 populations? Oecologia, 176, 595–605.
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545 **FIGURE CAPTIONS**

546 **Figure 1** Representative photomicrographs of HVC (**a**), Area X (**b**) and Rt (**c**) taken at 10 X magnification.

547

548 **Figure 2** Gonad mass for the two species at the different times of the year. We plotted the means for
549 the breeding season (March – April) and the rest of the year (August – December). Error bars represent
550 standard error. Asterisks indicate significant results (*$*p < 0.05, ** p < 0.01$*).

551

552 **Figure 3** Volumes for the different brain regions, plotted by species and season. We plot the means
553 for nuclei volume calculated as a percentage of telencephalon volume. **a.** HVC (mean $\pm$ SE): there is a
554 significant seasonal difference in the great tit HVC, but not in the willow tit HVC; **b.** Area X (mean $\pm$
555 SE): there is no seasonal difference in the great tit or the willow tit Area X; **c.** Nucleus Rotundus (mean
556 $\pm$ SE): there is no seasonal difference in the great tit or the willow tit Rotundus, but the great tit
557 Rotundus is significantly larger than the willow tit Rotundus. Asterisks indicate significant results (*$*p$
558 $< 0.05, ** p \leq 0.01$*).